FIBROUS FROM GLOBULAR PROTEINS[1]

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Most proteins of current or potential industrial importance are n the molecular sense compact, almost globular structures as they occur in nature or as they are isolated by laboratory or commercial procedures. While the structure of none of the proteins is known in detail, it is a widely held view that, fundamentally, the molecules are chainlike (1, 12, 25) but are normally maintained in a coiled configuration by interaction among the numerous polar groups. Opportunity for interaction between molecules is thus minimal. If the molecules were uncoiled and fixed in a linear form, the number of intermolecular bonds would be far greater, and the mechanical properties of objects such as fibers, films, and plastics made from the altered proteins should be much improved. This is not merely conjectural, for filaments of the natural fibrous proteins, silk and collagen, are far stronger and tougher than filaments of unoriented globular proteins. Casein fiber is now in commercial production, and experimentation is being conducted in several laboratories on the preparation of fiber from soybean and peanut proteins, zein, and other globular proteins. Fiber prepared in the conventional way from an alkaline dispersion has in general been somewhat deficient in dry and wet strength and gives no evidence of molecular orientation in its diffraction pattern. It is the purpose of this paper to describe experiments by which conversion of several globular proteins to the fibrous form has been accomplished and to give some of the properties of filaments made of the converted protein.

Observations of Carothers and Hill (9), Sookne and Harris (27), Mark (19), and others indicate that the length of an extended linear polymer molecule, as measured by the average degree of polymerization (D. P.) or molecular weight (M. W.), must exceed a critical minimum if fibers of usable pliability and strength are to be prepared. For the polyester of ω-hydroxydecanoic acid, Carothers and van Natta (10) found the limit to be between M. W. 10,000 and 15,000 or D. P. about 60 to 90. This corresponds to a length of 800 to 1200 Å. For cellulose and for polyamides the figures are roughly the same, while for hydrocarbons the D. P. for corresponding tensile strength is considerably higher because of the weakness of the interaction between non-polar groups. In all these linear polymer molecules, side groups are either completely absent or, if present, are small, are alike, and are spaced at regular intervals along the chain. On stretching, the molecules are readily oriented and form quasi-crystalline arrays in which segments of adjacent molecules match one another rather perfectly. Within

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these crystalline regions the potential energy tends toward a minimum, and intermolecular cohesion is correspondingly increased.

The molecular weight of globular proteins ranges from about 15,000 to several million (29). As measured, it indicates the weight of the effective kinetic units in sedimentation, osmosis, or diffusion, or the weight of crystallographically equivalent constituents in the unit cell as determined by x-ray analysis. It does not necessarily give any indication of the molecular weight, the degree of polymerization, and the maximum length of the polypeptide chain or chains contained in the molecule, and for our purposes these are the significant quantities. For example, the molecular weight of hemoglobin is about 68,000. Of this, 2500 units are due to the four associated haems. On treatment even with as mild a reagent as aqueous urea, hemoglobin splits into halves of molecular weight approximately 34,000. From a partial structure analysis, Boyes-Watson and Perutz (8) have proposed that the hemoglobin molecule consists of four substantially equal and parallel layers of polypeptide chains, with the main chains folded in the plane of the layers and the side chains extending at right angles to them. The position of the haem groups is unknown. If this model is correct, it is evident that the upper and lower pairs of layers are not joined by a peptide or other strong bond. Whether the paired layers are themselves linked by a strong covalent bond is not certain. If they are not, one would expect to be able to split the pairs into halves of molecular weight 17,000. Thus far, there is no report that hemoglobin from the higher animals has been dissociated into fragments of this size, but the hemoglobin of the cyclostomata, containing one haem per molecule, has a normal weight of 17,000 (28).

Molecular-weight measurements of globin derived from normal (68,000 M. W.) hemoglobin are not helpful, because globin readily forms loose association complexes and apparently it is the weight of the complex rather than the weight of the individual molecule that is usually found (24). Chibnall (11) has recently presented what seems to be good evidence that hemoglobin (68,000 M. W.) contains sixteen peptide chains. These may or may not be of similar size and constitution. From what has been said above, it is probable that not more than eight chains are firmly bonded together. If eight chains were joined by strong bonds adjacent to terminal amino groups to make a continuous chain of weight 34,000, the latter would have a D. P. of about 320, and if extended would be 1050 Å. long.[3] This should make a satisfactorily strong, tough fiber. It is important to note that in globular proteins about half the total weight is in the side chains, so that a fully extended 34,000 M. W. molecule would be much shorter than a molecule of rubber, 6-6 nylon, or polyvinyl alcohol of equal molecular weight, but would be comparable in length to cellulose. The effect of smaller D. P. and chain length on intermolecular cohesion may, however, be partly or fully compensated for by the large proportion of polar groups in both the main

[3] The average residue weight for globin is 106 (Chibnall (11)). Dividing 34,000 by 106 gives for the D.P. a rough value of 320. The length of the extended chain is computed by multiplying D.P. by 3.3 Å., the fiber repeat distance found in β-keratin and in globular proteins converted to the fibrous form.

and the side chains. If the chains pack regularly, as they do in collagen, a strong structure results.

Should the eight polypeptide chains in the hemoglobin half-molecule be linked, not end-to-end, but through cross-bonds, the length of fully extended chains would be correspondingly shorter, and conceivably no usably strong fiber could be made from hemoglobin regardless of how perfectly the chains might be oriented and packed. Taking an extreme case of sixteen equal polypeptide chains without covalent links, the D. P. and maximum length of each chain would be about 40 and 120 Å., respectively. These values are much smaller than the minimum values for usable fibers quoted earlier.

Chibnall (11) has concluded similarly that molecules of edestin (M. W. 310,000), insulin (35,000), ovalbumin (43,000), and lactoglobulin (42,000) are systems of six, eighteen,[4] about four, and nine peptide chains, respectively. It is well known that edestin in urea solution splits into fragments having a molecular weight of about 50,000. Apparently, then, edestin contains single peptide chains having a weight of 50,000, amply long for our purposes if they are not cross-bonded or degraded.[5] For the other proteins listed, no reliable estimates of the effective D. P. can be made, since the mode of linking of the constituent peptide chains is unknown. It is clear, however, that for these globular proteins, which may probably be regarded as typical, the highest possible D. P. does not greatly exceed the permissible minimum for forming fibers of good quality. As a corollary it follows that the process of unfolding and orienting the chains, and all other procedures applied to the protein, may have to be mild if ruinous chain degradation is to be avoided.

Astbury, Dickinson, and Bailey (2), working with edestin, first described the conversion of a globular protein to the fibrous form. By a different method Palmer and Galvin (22) and Lundgren and O'Connell (18) produced the same result with ovalbumin. Through work done in this laboratory still other means have been developed for converting globular proteins to a fibrous form in which the peptide chains are unfolded, oriented, and regularly arranged in space. One such means (21, 26) involves, essentially, heating the protein in the presence of water,[6] followed by mechanical treatment such as stretching to extend and orient

[4] Chibnall, taking 35,000 for the molecular weight of insulin, computed that there are eighteen chains in the molecule. The recent work of Miller and Anderson (J. Biol. Chem. **144,** 459 (1942)) indicates that the molecular weight of insulin is actually about 46,000. By using this value rather than 35,000, the number of polypeptide chains per molecule is raised to twenty-four.

[5] Adair and Adair (Biochem. J. **28,** 199 (1934)) suggest from mesasurements of membrane potentials that edestin on mild acid hydrolysis is split into fragments of molecular weight approximately 17,000. Bailey (Biochem. J. **36,** 140 (1942)) has found the sedimentation constant of a similar edestin preparation to be 2.6×10^{-13}. Correction for electroviscosity would raise this value and would indicate that the molecular weight of the sedimenting particles is roughly 40,000 to 50,000. If the molecular weight were 17,000, the sedimentation constant would be about 1.8×10^{-13}, or less if there were appreciable electroviscosity. It appears to us that there is no conclusive evidence that the edestin submolecule of M. W. 50,000 breaks down into 17,000 M. W. fragments.

[6] It has been found that heat as an agent in effecting the transformation from the globular to the fibrous state may be supplanted by a variety of chemical agents. A brief report of

the chains. Primary evidence that the conversion has been accomplished is provided by x-ray observations. The processed protein gives a typical "fiber diffraction pattern" which is remarkable in its similarity to the fiber pattern of the β-keratin of feathers and stretched hair. Among the globular proteins which we have thus converted to the oriented fibrous form giving the β-keratin diffraction pattern are casein, lactoglobulin, zein, hemoglobin, soybean protein, peanut protein, ovalbumin, edestin, tobacco-seed globulin, pumpkin-seed globulin, gliadin, and the albumin, globulin, and mixed proteins of horse serum.

PREPARATION OF SPECIMENS

Coarse fibers or bristles made by extrusion were convenient specimens for orientation. Suitable mixtures for extrusion were made from the more soluble proteins by mixing the powdered material with approximately half its weight of water. Pumpkin-seed globulin and other water-insoluble native proteins were tackified by incorporating about 1 per cent of sodium chloride with the protein–water mixture. Casein filaments were extruded at 95°C. from a briefly heated mixture of casein and water. Zein and gliadin were precipitated from alcoholic solution by addition of water. After washing with water, they were readily extrudable.

Thin sheets, plates, and small rods formed by conventional methods were also used as specimens. In making the plates, forming and heat treating were done simultaneously in a press. The heat treatment was usually given close to 100°C. Filaments of readily denaturable proteins such as lactoglobulin, serum albumin, the oil-seed globulins, and ovalbumin were simply kept in boiling water for 5 to 30 min. Filaments of casein, zein, and gliadin were conditioned in water vapor at 0.5–1.0 atmosphere pressure for several hours. During the heat treatment the filaments become tough and rubbery and so coherent that they may in general be stretched a considerable amount before fracture occurs. Changes in molecular structure that take place simultaneously will be discussed in the section on diffraction effects. Since the heat-conditioned proteins are decidedly viscous, application of shear stress provides the most obvious means of producing molecular orientation. Stretching has been utilized almost exclusively, because it is a convenient technique and because it is possible without undue difficulty to

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initial observations has already been made (21). Of the several proteins thus far examined, ovalbumin has been outstanding in the variety of reagents found that may substitute the thermal treatment. As judged by several tests, the mode of interaction of protein with both heat and the chemicals is similar. Ovalbumin filaments soaked in, for example, 75 per cent ethanol, or in boiling water become insoluble, elastic, and structurally so composed that they can be stretched to several times their initial length. The stretched filaments have much increased tensile strength and are pliable when dry. They are positive-birefringent and give β-keratin fiber patterns.

A specimen of ovalbumin surface denatured in a Waring Blendor, when stretched, gave the β-keratin diffraction pattern. An unusual feature of this pattern was the occurrence on the equator of a reflection at about 23 Å.

Ovalbumin, dispersed in phenol and spun into a precipitating bath such as ethanol, also gave a fiber pattern after stretching.

prepare specimens sufficiently cohesive to permit stretching. Stretching was usually done slowly in steam or in air approximately saturated with water vapor at about 100°C. Draw ratios (D. R.) or ratios of final to initial lengths of five or more were commonly obtained. In general, molecular orientation, as revealed by the diffraction pattern, increased with the draw ratio under comparable conditions. When specimens were drawn at room temperature or at an elevated temperature with a low pressure of water vapor, a small draw ratio produced the same diffraction effects as a much higher draw ratio when the specimens were more highly plasticized. On stretching in an alkaline solution or in the vapor of a boiling saturated solution of cresol, draw ratios as high as 25 have been obtained without any comparable increase in orientation.

Attempts to produce orientation in the absence of water have uniformly failed. Specimens prepared, for example, from intensively dried ovalbumin and cresol were stretched in cresol vapor at temperatures up to 150°C. or more. The diffraction pattern of the stretched material consisted of two full, diffuse rings.

Since the orientation process appears to be rather generally applicable to globular proteins, it is perhaps not surprising that proteins of various degrees of purity and from various sources should when oriented give comparable diffraction effects.[7] In our experience there is, however, a marked variation in the ease with which the different proteins and protein preparations may be crystallized and oriented. (The word crystallized is used in the restricted sense that the x-ray reflections sharpen and become more numerous, indicating increased regularity or order in the spatial arrangement of the scattering matter.) Generally speaking, the proteins ordinarily classed as heat-denaturable are most readily converted to the oriented fibrous form by the thermal process. Examples of readily convertible proteins are lactoglobulin, serum albumin, ovalbumin, and the globulins of tobacco and pumpkin seeds, provided that these are in the native state. If the proteins are denatured, as by heating, prolonged drying, chemical agents, or other means, the conversion becomes more difficult and less complete. The increased difficulty probably arises largely from insolubilization and loss of tack, making it hard to form filaments or other objects suitable for stretching. We have not yet sought to determine the feasibility of producing an oriented fibrous protein from a denatured protein powder by other shear processes, such as rolling, or flowing, or extruding under high pressure. Casein, zein, gliadin, and commercial soybean protein have proved considerably more difficult to crystallize and orient than the native, heat-denaturable proteins listed above.

DIFFRACTION EFFECTS

In our experiments, we have been concerned with the conversion of the globular protein molecule to an extended polypeptide chain in filaments (or other

We should like to express our thanks to the following individuals and organizations for making available available some of the proteins used in this work: The Drackett Company for soybean protein; W. G. Gordon for β-lactoglobulin; R. W. Jackson for edestin; the Northern Regional Research Laboratory for zein and gliadin; Sharp and Dohme, Inc., for serum proteins; the Southern Regional Research Laboratory for peanut protein; H. B. Vickery for gliadin and pumpkin- and tobacco-seed globulins.

formed objects). That is, the filaments as extruded are composed of native protein, and denaturation or unfolding of the molecules occurs in subsequent operations. X-ray diffraction measurements provide the best method of following the change in molecular configuration that takes place during these operations.

The diffraction pattern of most protein filaments as extruded and dried at room conditions without further treatment is characterized by two diffuse reflections, corresponding to interplanar spacings of about 10 Å. and 4.6 Å. The breadth of these reflections, the fact that only two reflections are observed, and the general scattering on the pattern indicate that the size of any crystalline regions in the sample must be very small. The spacings 10 Å. and 4.6 Å. must correspond to frequently occurring interatomic distances, and by comparison with x-ray patterns of disoriented keratin have been identified as the "side-chain" and "backbone" spacings between adjacent polypeptide chains (3). That is, considerable portions of the polypeptide chains form mesomorphic regions in which a number of chain segments lie parallel over a distance corresponding to several amino acid residues. This does not necessarily imply that the chains are unfolded; adjacent segments in a given region may be part of one more or less regularly folded protein molecule. The pronounced broadening of the two observed reflections is probably best interpreted as due to imperfect side-by-side packing of the amino acid residues rather than to extremely small perfect crystallites.

When a globular protein is heated with water, the diffraction pattern improves considerably. The 10 Å. and 4.6 Å. reflections sharpen, and at least one additional reflection (3.7 Å.) appears. Astbury and Lomax (3) have observed this change in the patterns of serum albumin and egg white upon heat denaturation and have attributed the pattern to regular bundles or crystallites composed of fully extended parallel polypeptide chains. We have found that filaments of many globular proteins heated to give the pattern characterized by three or more well-defined reflections can, in general, be stretched when plasticized with water to give a diffraction pattern nearly identical with that of β-keratin. On stretching, the existing crystallites are oriented with their longest axis parallel to the direction of stretch, and possibly new crystallites are formed by the action of the stress in extending and parallelizing polypeptide chains. The formation of crystallites or ordered regions of extended chains from curled or folded chains in the protein filaments is analogous to the ordering that occurs in the cold-drawing of polyamide fibers (20).

The rate of transformation of the protein from the mesomorphic state to the more ordered state under the influence of heat and water depends on the particular protein. Thus proteins readily denatured by heat, such as ovalbumin, lactoglobulin, and hemoglobin, are converted to the ordered state after a few minutes in boiling water. Since insolubilization of these proteins occurs rapidly on contact with the boiling water, there is no disintegration or dissolution of the filaments. Filaments of casein, on the other hand, are not readily heat-denatured, and if immersed in boiling water after extrusion, they rapidly disintegrate before they are insolubilized in the conversion to the ordered state. Consequently,

protein filaments of this type must be treated by a different method, and it has been our practice to heat them in atmospheres of controlled humidity. For example, casein fibers are conditioned at 100°C. over a 5 per cent solution of sodium chloride. There is a progressive change in the diffraction pattern of casein filaments with increasing conditioning time. The 4.6 Å. reflection becomes sharper, and a new reflection at 3.7 Å. is resolved. At later stages, two weak reflections at 2.2 and 2.0 Å. appear.

Although the x-ray pattern generally shows continuous improvements with increased time of heat-conditioning, mechanical properties of fibers may be impaired by long heating. Casein bristles, for example, show a decline in tensile strength if heat-conditioned for more than 50 hr. under the conditions described above. This may be due to chain degradation, which would not hinder the ordering process but would reduce the tensile strength.

We have observed that the water content of the protein filament has an effect on the x-ray pattern. As an illustration, wet casein gives sharper reflections than does bone-dry casein. To study this effect in greater detail, two series of freshly extruded casein filaments were equilibrated at 27°C. in air of 0, 25, 50, 75, and 100 per cent relative humidity. In one series, equilibrium was approached from the dry side and in the other series from the wet side. The equilibrated filaments were mounted in a closed chamber which maintained the proper humidity during exposure to the x-ray beam. In each series, equilibrating the filaments at 50 per cent relative humidity or greater sharpened the reflections, but there was little or no effect below 50 per cent relative humidity. Most of our patterns have been obtained from filaments equilibrated at room conditions, which correspond to 15 to 30 per cent relative humidity. Moreover, the sharpening of the pattern due to water alone is much less than the corresponding change resulting from heat-conditioning, so there is no doubt that heat-conditioning has a real effect on the crystallinity of the protein.

The action of heat in rearranging the mesomorphic structure in quenched polyamides (13) and cellulose triesters (6) to give a crystalline structure indicates that there is high mobility of chains or chain segments in these substances in the solid state. The crystallinity of quenched cellulose derivatives (6) which contain unsubstituted hydroxyl groups cannot be improved by annealing at temperatures below the softening point. The reduced mobility of the chains in these derivatives has been attributed to the presence of hydrogen bonds between residual hydroxyl groups. If such derivatives are annealed in water at 100°C., however, the strong hydrogen-bonding agent, water, splits the interchain hydrogen bonds and permits chain motion and crystallization.

Proteins contain a high concentration of hydrogen-bonding and other polar groups which contribute to a high interaction between adjacent chains. One might predict that there would be little tendency for chain rearrangement upon the application of heat alone, and that it would be necessary to introduce some polar solvent to increase mobility. This is borne out by the observations of Barker (7) that ovalbumin dried over phosphorus pentoxide is denatured, as measured by solubility, only at 160–170°C., while ovalbumin in the presence of excess water is denatured at 56°C. We have tried to crystallize and orient

globular proteins by our usual techniques, substituting for water other representative hydrogen-bonding liquids such as ethanol, cresol, and formamide. Our attempts have been uniformly unsuccessful.

TABLE 1

Observed spacings and intensities in the fiber diagram of lactoglobulin, ovalbumin, and β-keratin

INDEX	LACTOGLOBULIN	OVALBUMIN	β-KERATIN*
	Å.	Å.	Å.
001	9.7 S	9.8 S	9.7 S+
200	4.6_8 S+	4.6_5 S+	4.65 S+
400		2.3 W	2.4 W
600		1.6 W	
111	4.7 M	4.7 M	4.7 M
210	3.7_2 M+	3.7_5 M	3.75 S
410		2.2 W	2.2 W
020	3.2_6 M+	3.3_3 M+	3.33 S
220	2.7_1 W	2.7_5 W	2.7 W
030			2.2 W
130	2.1_6 W	2.1_8 W	
230	2.0_3 W+	2.0_3 W+	2.0 W

* From reference 4.

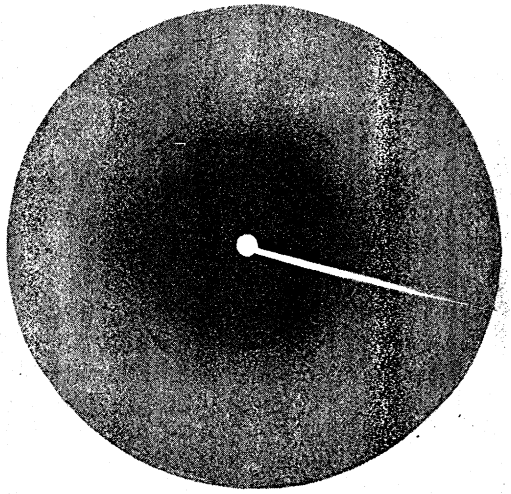

FIG. 1. X-ray diffraction pattern of a highly oriented ovalbumin filament. The fiber axis is vertical.

Protein filaments, annealed and stretched, give x-ray fiber patterns very nearly identical with the pattern of oriented β-keratin. Patterns showing the greatest number of reflections have been obtained from β-lactoglobulin and ovalbumin. Data on these proteins are summarized in table 1, together with indices and corresponding data for β-keratin given by Astbury and Street (4). The diffraction pattern of a highly oriented ovalbumin filament is shown in figure 1.

The 2.2 Å. reflection indexed by Astbury and Street as (030) is resolved into two near-meridian reflections which we have designated as (130). Thus, both (010) and (030) are absent, and the b-axis is probably a twofold screw axis. This is consistent with the packing of extended chains, with side chains projecting on alternate sides of the main chain, in successive amino acid residues. Such a structure is required by a fully extended polypeptide chain of amino acid residues having the same configuration (14). Meridian reflections at distances greater than 3.3 Å., such as are observed in feather keratin, do not occur in any of our preparations and, so far as we are aware, have not been found in the β-keratin of stretched wool and hair.

Comparison of the lattice spacings of β-lactoglobulin, ovalbumin, and β-keratin in table 1 shows only small variations, which are probably within the experimental error of measurement. Our data on oriented filaments of casein, pumpkin-seed globulin, hemoglobin, serum albumin, zein, gliadin, tobacco-seed globulin, edestin, soybean and peanut protein, while not as extensive as those for

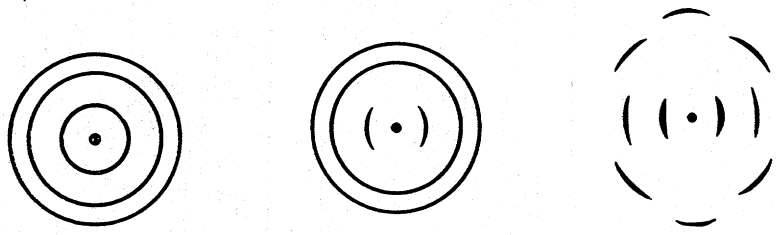

FIG. 2. Change of the diffraction pattern of a heat-conditioned globular protein during orientation by stretching: (a) no stretch; random orientation of crystallites; (b) low stretch; uniplanar orientation; (c) high stretch; uniaxial orientation.

ovalbumin and β-lactoglobulin, also show that the lattice spacings of these proteins are close to those of β-keratin.

Starting with an unstretched filament of denatured protein which gives an x-ray pattern corresponding to random orientation of crystallites, there is a particular sequence in which the three strongest x-ray reflections arc as stretching proceeds. This sequence is illustrated in figure 2. At low elongations, the innermost reflection (10 Å.) shows a concentration of intensity on the equator of the pattern, while the other reflections appear to have a uniform distribution of intensity. At higher elongations the 4.6 Å. reflection shows an intensity maximum on the equator, and the 3.7 Å. reflection shows four intensity maxima at about 45°. This phenomenon must be related to the shape of the crystallites or ordered regions which are being oriented by the stretching of the fibers.

A model which seems to account well for the observed phenomena may be described as follows: We may consider the crystallites to be plates of dimensions x, y, and z, where $x > y >> z$. The polypeptide chains are assumed to be parallel to x; the 10 Å. spacing is approximately along z; the 4.6 Å. spacing, approximately along y; and the 3.7 Å. spacing, a diagonal spacing perpendicular to the direction z. In unstretched filaments the platelets have random orientation

(figure 3a). Upon application of stress the first tendency will be for the plane xy to orient in the direction of stress, that is, the fiber axis, but with no preferential orientation of x or y of the various platelets (figure 3b). Orientation of the planes xy in the direction of stress requires the lattice planes with the 10 Å. spacing to be parallel to the fiber axis and hence to reflect on the equator. Since the planes xy have random orientations around a perpendicular to the fiber axis, the lattice planes of spacing 4.6 Å. and 3.7 Å. will reflect with uniform intensity through 360° on the x-ray photograph. Upon further stretching (figure 3c), the longest axis x of the platelets will turn toward the direction of stress, orienting the 4.6 Å. planes parallel to the fiber axis, and causing the corresponding reflection on the x-ray photograph to be arced on the equator. Similarly, the diagonal planes of 3.7 Å. spacing will give four intensity maxima at approximately 45°.

According to the structural scheme proposed by Astbury and Woods (5), the 10 and 4.6 Å. spacings are lateral spacings of the polypeptide chains and are ap-

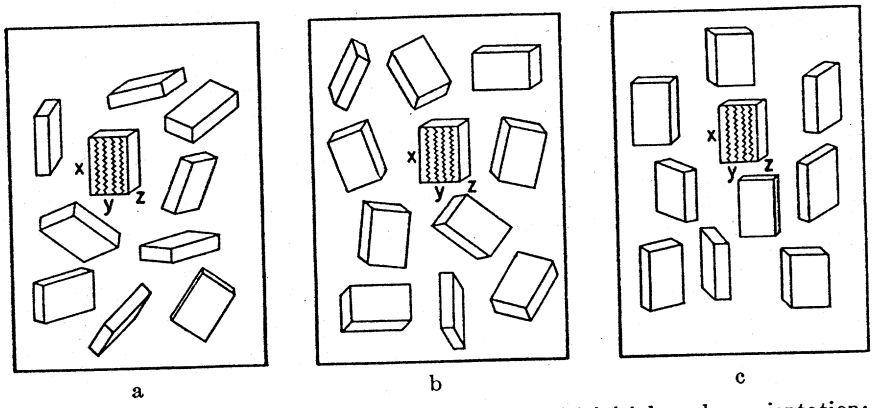

FIG. 3. Orientation of crystallites during stretching: (a) initial random orientation; (b) uniplanar orientation; (c) uniaxial orientation.

proximately at right angles to each other. From changes observed on swelling, Astbury and Lomax (3) concluded that the 10 Å. spacings are in the direction of the side chains, while the 4.6 Å. spacing represents the separation of the chains in the plane of the zigzag of the main chain, as illustrated in figure 4.

From the orientation sequence, then, the crystallites must be much longer in the direction of the backbone spacing than in the direction of the side-chain spacing. This conclusion is supported by the breadth of the corresponding reflections, the 10 Å. reflection being much broader than the 4.6 Å. reflection. Assuming equal lattice perfection in the two directions, this indicates that the crystallite is much smaller in the 10 Å. or z direction. Additional evidence is supplied by specimens which have been rolled to give biaxial orientation of the crystallites. X-ray patterns of such specimens taken with the beam parallel and at right angles to the plane of rolling are shown in figure 5.

It is seen that the crystallites orient so that the planes with the 10 Å. spacing are parallel to the plane of rolling, that is, the z direction of the crystallites is per-

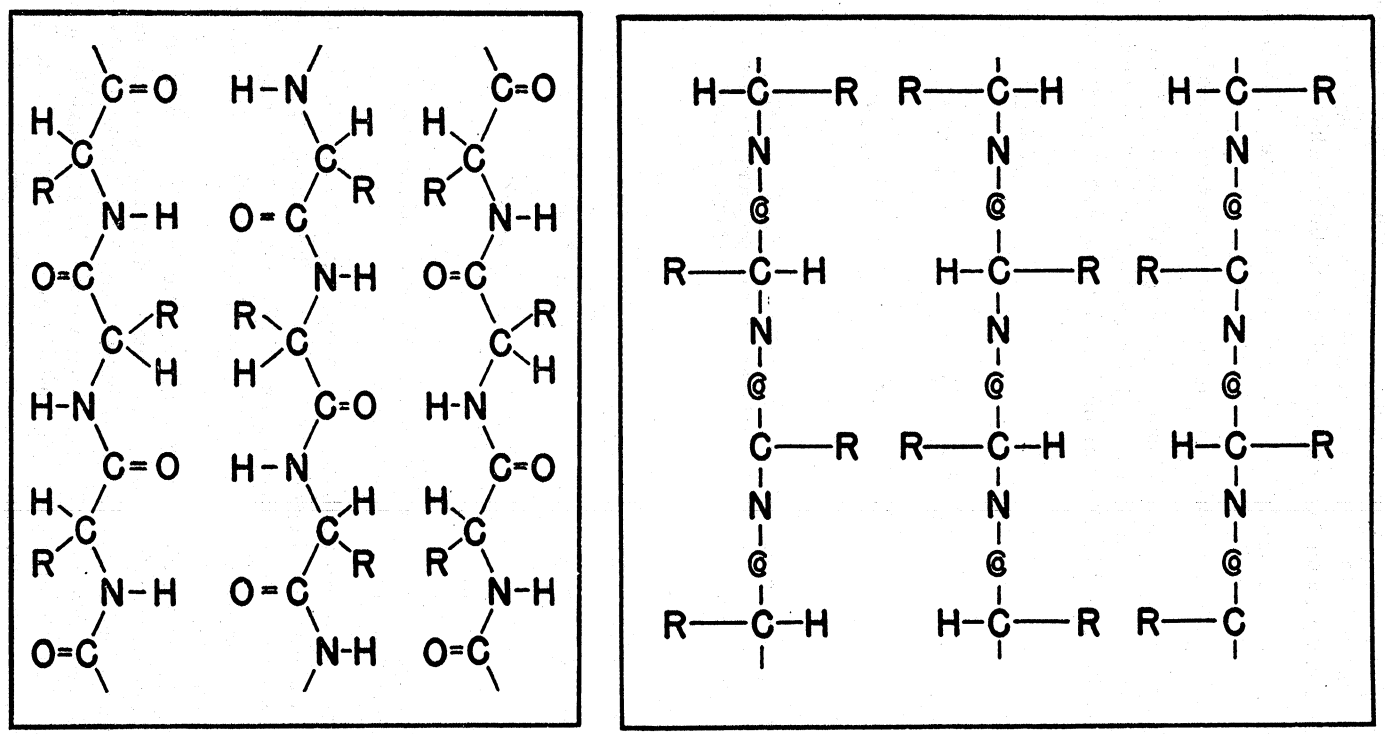

FIG. 4. Packing of extended peptide chains in the direction of (a) the 4.6 Å. "backbone" spacing, and (b) the 10 Å. "side-chain" spacing. Hydrogen atoms attached to nitrogen atoms are not shown.

pendicular to the plane of rolling, while the x and y directions lie in this plane. This, again, is consistent with the relative lengths $x > y >> z$. The crystallites thus have a pronounced tendency to grow in the direction in which the polypeptide chains are bonded laterally by hydrogen bridges between $\rangle C{=}O$ and $\rangle N{-}H$ groups of adjacent chains. The regular occurrence of these groups along the chain gives opportunity for regular packing of chains in the direction of the hydrogen bridges. On the other hand, the packing in the direction of the side chains will be influenced by the variable length and nature of the side chain

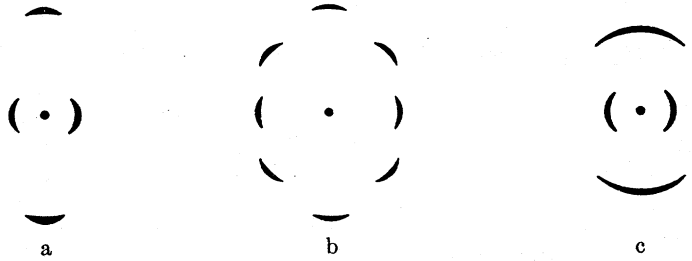

FIG. 5. Diffraction patterns of biaxially oriented ovalbumin. (a) X-ray beam perpendicular to the fiber axis, parallel to the plane of rolling; (b) beam perpendicular to both fiber axis and plane of rolling; (c) beam parallel to fiber axis. Plane of rolling is vertical.

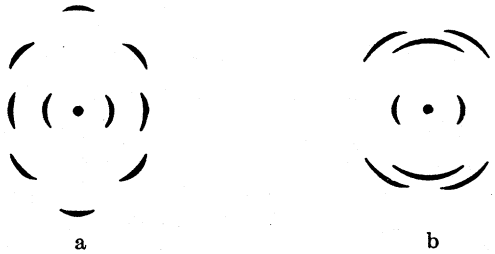

FIG. 6. The two types of fiber pattern exhibited by oriented globular proteins: (a) the "β-keratin" patterns; (b) the "egg-white" pattern.

from the different amino acid residues, and consequently the order will be much less perfect and will extend a shorter distance. The 3.3 Å. "fiber repeat" reflection is normally weak. It becomes prominent only in rather well-oriented specimens, even though the repeating units lie in the direction of the longest dimension of the crystallites.

The tendency of the crystallites to grow in the y direction may explain another type of diffraction pattern frequently observed in drawn protein filaments. This pattern, illustrated in figure 6, was first observed by Astbury, Dickinson, and Bailey (2) in stretched films of poached egg white. The distinguishing feature of this pattern is that the 4.6 Å. reflection is arced on the meridian rather than on the equator, the interplanar spacings of the three principal reflections being the same as in the β-keratin type. Astbury, Dickinson, and Bailey (2)

report a faint reflection of 3.3 Å. spacing on the equator, corresponding to the repeat distance along the polypeptide chain, but we have never observed this although we have examined dozens of photographs showing this egg-white pattern. The simplest explanation of this photograph is that the crystallites have developed such that $y > x > z$. Hence, upon stretching, y will orient along the fiber axis, causing the 4.6 Å. reflections to fall on the meridian, while the 10 Å. reflection occurs on the equator as before.

This pattern has been obtained from ovalbumin, casein, zein, peanut protein, hemoglobin, and edestin. It usually occurs if a fiber is given relatively low extension after a relatively short period of heat-conditioning. For example, it appears when ovalbumin filaments are immersed in boiling water for 2 to 5 min. and are then stretched 50 to 85 per cent. At greater elongations this pattern is transformed to the β-keratin pattern. The reverse transformation of the β-keratin pattern into the egg-white pattern has never been observed. Although many oriented protein filaments have been contracted in hot water or steam to give disoriented β-keratin, an intermediate form giving the egg-white pattern has not occurred.

BIREFRINGENCE

X-ray diffraction provides the best method of gaining information about the orientation and packing of the molecules in the crystalline regions of a fiber, but it gives little direct information concerning the alignment of chains in the less-ordered regions. Optical and swelling anisotropy do, however, provide a rough measure of orientation of intercrystallite chains.

The refractive index of a substance for plane polarized light depends on the polarizability of the atomic groups in the direction of the plane of the electric vector. When a high polymeric substance is stretched, there is an alignment of molecular groups which usually results in greater polarizability along the direction of stretch than at right angles to it, with a consequent difference in refractive index in these two directions. Birefringence has the advantage that it can detect orientation in substances which do not produce any easily observable x-ray diffraction effect and are thus seemingly amorphous. On the other hand, calculations by Treloar (30) indicate that the birefringence is insensitive to small departures from perfect orientation of crystallites along a given direction.

A comparative study of birefringence and diffraction effects has been made on ovalbumin fibers heat-denatured and stretched in steam. Birefringence was computed from the retardation and the diameter of the fibers. The retardation was measured by means of an uncalibrated quartz wedge. Birefringence changes with draw ratio, as shown in figure 7. At D. R. = 2, diffraction patterns of the same fibers show marked arcing of the 10 Å. reflection, but only very slight arcing of the 4.6 Å. reflection. This corresponds to approximately uniplanar orientation of crystallites, as suggested above, and judging from the x-ray pattern alone the filaments should be optically nearly isotropic. The birefringence is 6×10^{-3}, however, suggesting that there is preferred orientation of polypeptide chains in the direction of stretch. Treloar (30) predicts that in this region of low

orientation the birefringence will change most rapidly. Part of the birefringence may be contributed by preferentially oriented intercrystallite chains that do not appreciably affect the diffraction pattern. This would be analogous to the behavior of polymerized methyl methylacrylate when stretched. The polymer molecules are evidently linear, but it is difficult or impossible to pack them into regular arrays in space. Although when stretched the material becomes birefringent, the diffraction pattern shows very little asymmetry (15, 23). Similarly, a number of samples of casein textile fiber prepared in this laboratory by precipitation from an alkaline dispersion, followed by stretching and formaldehyde hardening, had birefringence ranging from 1 to 10 $\times$ 10^{-3} without any indication of arcing on the diffraction patterns.

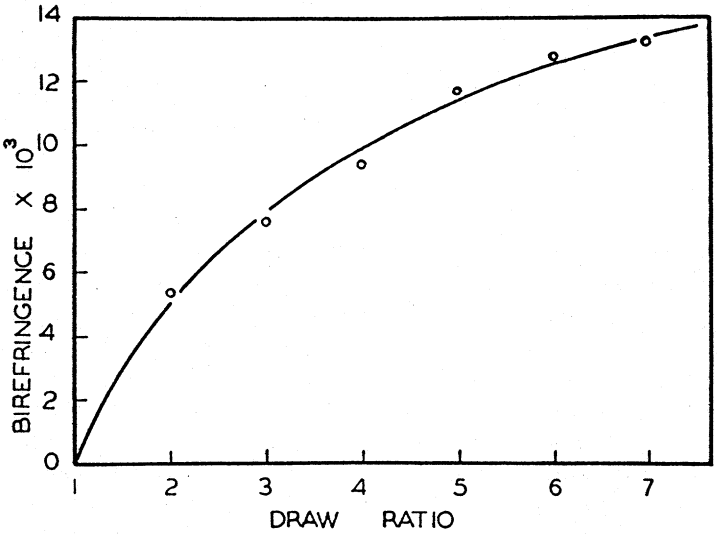

FIG. 7. Change of birefringence of ovalbumin with draw ratio

With increasing stretch, the birefringence of ovalbulin increases, while the arcs on the x-ray pattern decrease in angular width and increase in intensity. At D. R. = 7 the birefringence is 14 $\times$ 10^{-3}, and the slope of the curve of figure 7 indicates that it has not yet attained its maximum value.

It might be supposed that protein preparations giving the egg-white type of fiber pattern would be negative-birefringent. The refractive index is greatest along the direction of the extended peptide chains, and this direction in the crystallites from which the fiber pattern arises is perpendicular to the fiber axis. Actually the birefringence of all the several specimens examined was positive, and of the order of 10^{-3}. The following may be offered in explanation of this anomaly: By simple visual comparison of the intensity of arcs and rings, it is clear that the proportion of crystalline matter is not high. Furthermore, the diffraction pattern obtained with the beam parallel to the fiber axis has full rings of comparable intensity at 4.6 and 10 Å. In the diffraction pattern of the crystallites alone, the 4.6 Å. ring would be missing. Consequently, the negative birefringence con-

tributed to the total birefringence by the crystallites is small and is apparently overweighed by the positive contribution of the non-crystalline chains oriented parallel to the fiber axis.

High optical anisotropy without corresponding x-ray effects has also been found in fibers drawn from surface films. At a water–air interface, soluble globular proteins in general unfold to form a monolayer. This, on compression between parallel barriers, is anisotropic, and from it filaments may usually be drawn (17). Accepting the view of Langmuir (16) that protein monolayers are composed principally of long polypeptide chains, it might be expected that filaments pulled from such monolayers would contain the chains in a linearly extended parallel array. We have prepared fibers suitable for examination from monolayers of casein and ovalbumin spread on a 0.003 *M* citric acid–phosphate buffer at pH 4.7. At a pressure of approximately 25 dynes per centimeter, fibers were formed by slowly withdrawing a 0.5-mm. platinum wire from the film-covered surface. When maintained taut until air dry, many of the fibers had high positive birefringence, a maximum value of 7×10^{-2} being recorded. A bundle of fine ovalbumin fibers having a rather uniform birefringence of about 2×10^{-2} was examined in a micro-diffraction camera. The diffraction pattern consisted of full rings, with no suggestion of arcing. The combined evidence of birefringence and x-ray diffraction would seem to indicate that in the fibers there is a high degree of preferred orientation of chain molecules which are imperfectly packed. Because of the extreme smallness of the fibers, no attempt was made either to improve the spatial order by an annealing process or to measure the tensile strength.

SWELLING MEASUREMENTS

A stretched protein fiber dried under tension generally contracts in length when immersed in water, and contracts further upon drying. If the fiber is again immersed in water, both length and diameter increase. The contraction in length and the increase in diameter upon the first immersion in water diminish as the stretch given the fiber increases. For example at D. R. = 2 an unhardened ovalbumin fiber contracted 14 per cent in length and increased 33 per cent in diameter, while at D. R. = 8 the corresponding values were 3 per cent and 19 per cent.[8] Similarly, the reversible dimensional changes in a completely relaxed fiber decrease with elongation. At D. R. = 2 a relaxed fiber on wetting swelled 12 per cent in length and 20 per cent in diameter, while at D. R. = 8 the values were 4 and 13 per cent.

The fiber thus appears to be stabilized toward water by stretching. If stretching affected only the orientation of existing crystallites, we should expect that lateral swelling would increase and longitudinal swelling decrease. Instead, both longitudinal and lateral swelling decrease. This suggests that the molecular packing is improved by stretching, that is, crystalline areas are produced.

Swelling anisotropy is defined as the ratio of the increase in diameter to the increase in length of a fiber on immersion in a swelling liquid. For the ovalbumin

[8] These values refer to the wet fibers; on drying, further dimensional changes occur.

fibers of D. R. = 8 mentioned above, its value is slightly greater than 3. For cotton, wool, and silk, the value is about 10, whereas for regenerated cellulose it is about 6 to 8 (31).

TENSILE STRENGTH

As expected, tensile strength is increased upon conversion of globular proteins to the oriented fibrous form. Tensile measurements have been made for the most part on coarse filaments, approximately 150–300 microns in diameter. Single-filament breaking loads were measured on a Scott Type I-P-2 inclined-plane testing machine. Specimens were kept in the testing room at 70°F. and 65 per cent relative humidity at least 16 hr. before breaking. Initial diameters were used in the computations. Strengths reported are in general the average strength of ten or more fibers.

Air-dried extruded filaments of all the proteins were so weak and brittle that tensile measurements were impracticable, but after the heat treatment the strength[9] was 4.5 to 6.7 kg. per mm.^{2} Upon orientation, strengths were raised to the following values: casein, 18.6 kg. per mm.^{2}; pumpkin-seed globulin, 16.2; hemoglobin, 11.6; lactoglobulin, 12.8; zein, 17.6; ovalbumin, 20.9. Individual filament strengths as high as 30 and 34 kg. per mm.^{2} have been measured on specimens of casein and ovalbumin, respectively. All the oriented preparations gave β-keratin-like fiber patterns. It seems clear, however, that by no means all the protein even in our best specimens is in the form of oriented crystallites, although we know of no experiment by which the proportions of crystalline and non-crystalline material may be precisely judged.

On immersion in water, dimensional changes and changes in birefringence suggest that part of the protein in stretched filaments is unfolded and oriented but is not crystallized, and thus seems amorphous to x-rays. We are unable to estimate the proportion of molecules which have substantially the same configuration as in the original protein.

If the protein chain is 1000 Å. or so long when fully extended, two or more portions of the chain could pack in orderly arrays with matching segments of other protein molecules to form crystallites of a not improbable size—let us say 100–200 Å. Intercrystallite regions, although composed from the same chains forming the crystallites, may be incapable of forming a regular network in space. This may be caused by chain flexibility, variable chain length between crystallites, or other factors. On application of tensile stress to the system, the composite of crystallites and non-crystalline connecting matter tends to align itself parallel to the tension direction. Intercrystallite chains trend in the same direction. The chains are thus oriented, and in such a way as to increase the tensile strength of the whole structure, but there is no corresponding diffraction effect of discernible magnitude.

Protein filaments, crystallized and oriented by heat and stretching, contract

[9] Assuming the density of the protein filaments to be 1.29, the relationship between the common tensile strength units is as follows: 1 gram per denier = 11.6 kg. per mm.^{2} = 16,500 lb. per in.^{2}.

considerably in water. This property has been studied in some detail on ovalbumin, because high orientation can be produced more readily in it than in any other protein available in quantity with which we have worked. The crystallites in a highly oriented ovalbumin filament are rather stable structures, since the β-keratin diffraction pattern persisted even after the filament was boiled for 8 hr. in distilled water. During boiling, the crystallites are disoriented to a significant extent, as is evidenced by an increase in the angular width of the diffraction spots. The radial dimension and the sharpness of the spots are practically unchanged, however, indicating that the size of the crystallites and the perfection of atomic arrangement within them are largely unaltered.

On the other hand, oriented intercrystallite chains, being imperfectly packed, are readily penetrated by swelling agents such as water and forced apart, causing the specimen as a whole to contract unless it is clamped so that contraction is impossible. A fixing treatment, designed to minimize swelling by cross-linking chains or diminishing their hydrophilic character, should heighten the dimensional stability of the oriented fiber and increase its strength after swelling. Accordingly, oriented ovalbumin filaments were treated with acetic anhydride, with formaldehyde, and with benzoquinone.

In the acetylation experiments, filaments stretched in steam to the draw ratio 10.5 were heated with acetic anhydride at 100°C. for 1 hr. Residual acetic anhydride and acetic acid were removed by acetone in a Soxhlet extraction. To harden with formaldehyde, filaments with a draw ratio of 6.0 were soaked, while clamped, for 16 hr. at 25°C. in a solution containing 5 per cent formaldehyde and 30 per cent sodium acetate at pH 5.8. The filaments were washed thoroughly with water and dried for 1 hr. at 40°C. To harden with quinone, filaments with a draw ratio of 10.5 were soaked, while clamped for 40 hr. at 25°C. in a 1 per cent benzoquinone solution at pH 4.7 maintained by a 0.05 *M* acetic acid–acetate buffer. The hardened filaments were washed with water and dried at 40°C.

Tensile and retraction data on the treated and untreated oriented filaments are assembled in table 2. It is apparent that all three treatments markedly diminish dimensional change and reduction in strength on soaking in water, although the effect of formaldehyde is destroyed rather rapidly at 100°C. The fiber diagrams of acetylated and quinone-hardened filaments changed remarkably little, even on boiling for 4 or 8 hr. There is a small increase in the length of the arcs, corresponding to a slight disorientation of the crystallites, but otherwise the pattern is not altered. After corresponding treatment of formaldehyde-hardened oriented ovalbumin, the diffraction pattern was much like that of the unhardened protein. The principal reflections of the β-keratin structure were present, and the width and sharpness of the spots were about the same as they were initially, but the length was increased several fold.

Wet and dry strengths have been determined for a series of oriented coarse filaments. Before testing, the filaments were relaxed in water at 25°C. for 6 hr., dried at 40°C. for 1 hr., and stored at 70°F. and 65 per cent relative humidity for 16 hr. In addition, filaments used for wet-strength determinations were soaked in distilled water at 70°F. for at least 30 min. immediately before the tests were made. Table 3 gives the results of the tests. The data show that, while

TABLE 2

Effect of hardening by acetylation, formaldehyde, or quinone on the tensile strength and retraction in water of oriented ovalbumin filaments

(Measurements made on air-dry material)

Acetylation-hardened filaments soaked in water at 100°C.

	RETRACTION IN WATER, PER CENT				TENSILE STRENGTH, KG. PER MM.²				
	1 hr.	2 hr.	4 hr.	8 hr.	0	1 hr.	2 hr.	4 hr.	8 hr.
Hardened	10	11	12	16	21	14	13	12	9
Untreated	29	31	31	33					

Formaldehyde-hardened filaments soaked in water at 25°C.

	RETRACTION IN WATER, PER CENT			TENSILE STRENGTH, KG. PER MM.²			
	10 min.	1 hr.	24 hr.	0	10 min.	1 hr.	24 hr.
Hardened	5	6	6	16	16	15	16
Untreated	13	13	16	14	13	12	12

Formaldehyde-hardened filaments soaked in water at 100°C.

	RETRACTION IN WATER, PER CENT					TENSILE STRENGTH, KG. PER MM.²					
	10 min.	30 min.	2 hr.	4 hr.	8 hr.	0	10 min.	30 min.	2 hr.	4 hr.	8 hr.
Hardened	16	29	34	40	40	16	14	9	9	8	7
Untreated	36	39	40	44	46	14	7	7	7	7	7

Quinone-hardened filaments soaked in water at 25°C.

RETRACTION IN WATER, PER CENT			TENSILE STRENGTH, KG. PER MM.²			
10 min.	1 hr.	24 hr.	0	10 min.	1 hr.	24 hr.
0	0	2	16	19	17	19

Quinone-hardened filaments soaked in water at 100°C.

RETRACTION IN WATER, PER CENT				TENSILE STRENGTH, KG. PER MM.²				
10 min.	30 min.	2 hr.	4 hr.	0	10 min.	30 min.	2 hr.	4 hr.
5	5	7	9	16	16	15	16	13

TABLE 3

Tensile strength of relaxed oriented ovalbumin filaments

TREATMENT	DRY	WET	RATIO OF WET TO DRY STRENGTHS
	kg. per mm.²	*kg. per mm.²*	
Untreated	18.9	10.4	0.55
Acetylation-hardened	17.2	10.3	0.60
Formaldehyde-hardened	16.5	15.2	0.92
Quinone-hardened	17.8	14.9	0.84

the wet strength of oriented but unhardened filaments was only a little more than half the dry strength, the ratio for quinone- and formaldehyde-hardened filaments was increased to more than 0.8 and 0.9, respectively. On acetylation, the gain was slight.

SUMMARY

1. By means of heat, water, and mechanical shear, several globular proteins have been converted into a fibrous form giving an x-ray diffraction pattern nearly identical with that of β-keratin of stretched hair and wool. Among the proteins thus converted are lactoglobulin, ovalbumin, casein, zein, peanut protein, soybean protein, gliadin, hemoglobin, serum albumin, edestin, and tobacco-seed globulin. The process is most effective when applied to native, typically heat-denaturable proteins.

2. Under similar but milder conditions most of the proteins have given a second type of fiber pattern, characterized by the appearance of the 4.6 Å. "backbone" reflection on the meridian and the 10 Å. "side-chain" reflection on the equator.

3. When a protein is heated in the presence of water the diffraction pattern sharpens and new lines appear, indicating ordering of chains analogous to that occurring in the annealing of cellulose ester and polyamides.

4. Relative dimensions of the crystallites giving the fiber patterns are deduced from the sequence in which the x-ray reflections arc when a protein filament is stretched.

5. Comparative data on birefringence, x-ray diffraction, and swelling anisotropy as measures of molecular orientation are presented.

6. Tensile strength is increased by conversion of the globular protein to the oriented fibrous form. Wet strength after treatment with boiling water and dimensional stability of oriented ovalbumin filaments are improved by acetylation and hardening with formaldehyde or quinone.

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